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Molecular Phylogeny and Divergence Time Estimation of the Soft Coral *Dendronephthya gigantea* (Alcyonacea: Nephtheidae)

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ABSTRACT

Soft coral *Dendronephthya gigantea* (Verrill, 1864) is a conspicuous species dominating shallow sea waters of Jejudo Island, Korea. Recently its whole mitochondrial genome sequencing was completed by us and the sequence information provided an opportunity to test the age of Octocorallia and time of evolutionary separation between some representative orders of the subclass Octocorallia. Molecular phylogenetic analyses based on 13 mitochondrial protein encoding genes revealed a polyphyletic relationship among octocorallians representing two orders (Alcyonacea and Gorgonacea) and four families (Alcyoniidae, Nephtheidae, Briareidae, and Gorgoniidae). Estimates of divergence times among octocorallians indicate that the first splitting might occur around end of or after Cretaceous period (50-79 million years ago (Ma)). The age is relatively young compared to the long history of stony sea corals (>240 Ma). Taken together our result suggests a possible relatively recent radiating evolution at least in the order Alcyonacea and Gorgonacea. Molecular dating and phylogenetic analysis based on much broader taxon sampling and many genes might give an insight into this interesting hypothesis.

Key words: *Dendronephthya gigantea*, Octocorallia, molecular phylogeny, divergence time

INTRODUCTION

The soft coral *D. gigantea* (Verrill, 1864) is a dominant species around Jejudo Island, Korea. This species belongs to the subclass Octocorallia. Understanding of evolutionary relationships among octocorallians remained poorly until near past and still unresolved in most basal nodes of phylogeny. A few previous phylogenetic studies identified distinct clades within the taxonomic group, but some clades did not agree with the conventional taxonomy (Won et al., 2001). Won et al. (2001) found a lack of resolving power in 18S rDNA data within the subclass Anthozoa. Recently McFadden et al. (2006) assessed evolutionary relationships of octocorallians using two mitochondrial genes (ND2 and MSH1) from the largest taxon sampling (103 genera and 28 families) to date. This comprehensive study clearly showed an inconsistency between taxonomy and phylogeny, although basal relationships among major clades of octocorallians could not be resolved (McFadden et al., 2006). Largely difficulties of morphological classification in this group lie behind this discrepancy between taxonomy and phylogeny. On the other hand, application of molecular markers for phylogenetic analyses has been delayed in the Octocorallia due to dearth of appropriate markers. In particular evolution of mitochondrial DNA sequences in Anthozoa turned out to be extremely slow (Shearer et al., 2002). Thus mitochondrial genes lack sufficient resolution for discriminating species within genera (Sánchez et al., 2003; Cairns and Bayer, 2005; Wirshing et al., 2005). Nuclear 18S rDNAs did not provide resolution for deeper nodes within trees (Won et al., 2001). Recently Medina et al. (2006) applied whole mitochondrial genome to test evolutionary relationships among stony corals. Based on the phylogeny reconstructed from 13 different protein encoding genes, molecular dating of diverging ages at basal nodes was achieved. Because of large amounts of informative site in the long DNA sequences, the evolutionary relationship at the ordinal level between the order Scleractinia and Corallimorpharia could be clearly shown. However, more recently other research group who used small set of genes but larger taxon sampling found a contrasting result (Fukami et al., 2008). This conflict leaves further evaluation.

Our purpose in this study was to construct a well resolved phylogeny among octocorallians including the dominant soft coral *D. gigantea* and to estimate divergence times among them. We updated the earlier molecular data set made of most protein encoding genes of mitochondrial genome (Medina et al., 2006) with newly sequenced mitochondrial genome of *D. gigantea*. This new data set consists of main

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Table 1. Species and their mitochondrial genes used in the study

Species	GenBank	Notes*	References[†]
Octocorallia			
Dendronephthya gigantea	FJ372991	–	(Kim, 2008)
Pseudopterogorgia bipinnata	DQ640646	–	(Medina et al., 2006)
Sarcophyton glaucum	AF064823, AF063191	No sequences for ND3 and ND4	(Pont-Kingdon et al., 1998)
Briareum asbestinum	DQ640649	–	(Medina et al., 2006)
Corallimorpharia			
Corynactis californica	AB441341 for CYTB AB441256 for COI	Only available for CYTB and COI	(Fukami et al., 2008)
Ricordea florida	DQ640648	–	(Medina et al., 2006)
Discosoma sp.	DQ643965	–	(Medina et al., 2006)
Rhodactis sp.	DQ640647	–	(Medina et al., 2006)
Scleractinia "Complex" clade			
Porites porites	DQ643837	–	(Medina et al., 2006)
Siderastrea radians	DQ643838	–	(Medina et al., 2006)
Acropora tenuis	NC_003522	–	(van Oppen et al., 2002)
Pavona clavus	DQ643836	–	(Medina et al., 2006)
Agaricia humilis	DQ643831	–	(Medina et al., 2006)
Scleractinia "Robust" clade			
Astrangia sp.	DQ643832	–	(Medina et al., 2006)
Montastraea faveolata	NC_007226	–	(Fukami and Knowlton, 2005)
Mussa angulosa	DQ643834	–	(Medina et al., 2006)
Colpophyllia natans	DQ643833	–	(Medina et al., 2006)
Actiniaria			
Nematostella sp.	DQ643835	–	(Medina et al., 2006)
Metridium senile	NC_000933	–	(Beagley et al., 1995)
Zoanthiniaria			
Palythoa sp.	DQ640650	No sequences for CYTB, ND5, ND1, ND3, COII, ND4, and ND6	(Medina et al., 2006)

*Unless noted otherwise in the column, all the sequences from the thirteen protein encoding genes were included for phylogenetic analyses: three cytochrome *c* oxidases (COI, COII, COIII), cytochrome *b* (CYTB), and 7 NADH dehydrogenases (ND1, 2, 3, 4, 4L, 5, 6), and ATP synthases (ATP6 and ATP8).

[†] Original publications that reported and deposited the mitochondrial genome sequences.

taxonomic representatives of anthozoan groups which are closely related to octocorallians.

MATERIALS AND METHODS

Phylogenetic Analysis

We conducted phylogenetic analyses using thirteen mitochondrial protein encoding genes from 20 taxa including *D. gigantea*. Recently we completed mitochondrial genome sequencing of *D. gigantea* and deposited the sequence information to GenBank (accession no. FJ372991) (Kim, 2008). DNA sequences of the other 19 taxa representing Hexacorallia (Scleractinia, Corallimorpharia, Actiniaria, and Zoanthiniaria) and Octocorallia were collected from GenBank (Table 1). The single largest data set on whole mitochondrial genomes of anthozoans was published by Medina et al. (2006). We included those sequences in the present phylogenetic analyses. Alignments of the sequences were generated automatically and then edited manually based on amino acid translation in MEGA v.3.1 (Kumar et al., 2004). Ambiguous sites in homology due to indels were manually excluded and sites with gaps were included unless over half of the taxa are missed in the sites. A single frame of amino acid translation was applied to the concatenated data so that a data partition according to codon positions is easily made for Bayesian analysis. The final data set resulted in a concatenated sequence of 11,946 nucleotides (Table 2). To infer evolutionary relationships among the taxa, maximum likelihood (ML) trees and Bayesian trees were searched by PAUP v. 4.10b (Swofford, 2002) and MrBayes v. 3.12 (Huelsenbeck and Ronquist, 2001), respectively. The best-fit model of nucleotide substitution for ML search was chosen based on Akaike information criterion (AIC) using Modeltest program v. 3.7 (Posada and Crandall, 1998). According to the model testing, a general time reversible model (GTR) with a proportion of invariable sites (I) and gamma (Γ) distributed

Table 2. Thirteen protein encoding genes examined and their final length of DNA sequences for phylogenetic analyses

Gene	Length (n.t.)
COI	1,542
COII	744
COIII	786
CYTB	1,521
ND1	966
ND2	1,095
ND3	345
ND4	1,431
ND4L	300
ND5	1,761
ND6	558
ATP6	693
ATP8	204
All	11,946

rate variation among sites (GTR+I+ Γ model) was chosen for our data set. ML search was run under the following setting: tree bisection and reconnection (TBR) branch swapping with 100 random addition and 100 bootstrap replicates. Bayesian analyses were conducted under general time reversible model (GTR) with variable rate partitions for each codon position with gamma distributed rate variation among sites (GTR+SS+Γ model) as follows: Markov chain Monte Carlo (MCMC) with 2 million generations, 6 parallel chains, sampling every 1000 iterations, and burn-in 500. We used the last 1,500 sampled trees to estimate Bayesian posterior probabilities (BPP).

Estimation of Divergence Time

In order to estimates divergence times of each node of the phylogenetic tree, we used R8S v. 1.7 program by Sanderson (2002). The ML tree with branch lengths was used in the analysis of R8S. Because there is no known appropriate outgroup which has sequence information of all 13 protein encoding genes examined in the ingroup, we chose midpoint rooting method. A round of cross validation procedure tested log smoothing parameter value from 0 to 10, and found 1 the best parameter value for the data set. The value is sufficiently low to allow rate heterogeneity across branches. To calibrate divergence time we followed Medina et al.'s schemes (see Fig. 1, 2006): one from "robust" clade and the others from "complex" clade which represent two highly divergent clades of the order Scleractinia (Romano and Palumbi, 1997a, b; Chen et al., 2002). The former is genus *Astrangia* (70 Ma: an7 node in Fig. 1) and the latter are genera *Pavona* (33 Ma: an8 node) and *Acropora* (55 Ma: an9 node) (Fig. 1). Additionally a fossil record of the first appearance of Scleractinia at 240 Ma was set as a lower date boundary for the node an16 (Fig. 1). Labeling of the nodes included in the R8S analysis is represented in Fig. 1 and Table 3.

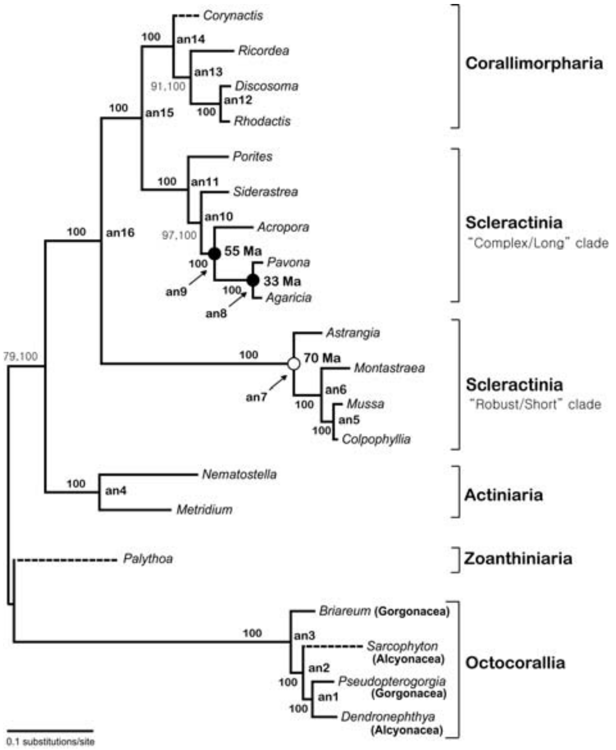

Fig. 1. Maximum likelihood (ML) tree of some representative anthozoans based on concatenated thirteen mitochondrial protein encoding genes (11,946 n.t.). The root of the tree indicates a midpoint of the longest branche. Lengths of branches are proportionally drawn to their substitution rates. Numbers above or below branches indicate bootstrapping values of ML and Bayesian posterior probabilities (BPP). If both values are equal, they are represented as a single value. Dashed lines indicate taxa that have incomplete protein encoding genes. Names of each node are also placed between two bifurcating branches from "an1" to "an16" in series. Fixed divergence ages based on earliest fossil records are shown at nodes indicated by open circle for *Astrangia* (70 Ma) of the "robust" clade and by filled circles for *Pavona* (33 Ma) and *Acropora* (55 Ma) of the "complex" clade of Scleractinia.

RESULTS AND DISCUSSION

We conducted maximum likelihood and Bayesian phylogenetic analyses involving the newly sequenced temperate soft coral species, *D. gigantea* (Alcyonacea: Nephtheidae), and other 19 anthozoan taxa representing Hexacorallia (Scleractinia, Corallimorpharia, Actiniaria, and Zoanthiniaria) and Octocorallia (Fig. 1). The phylogenetic analyses resulted in the same tree topology between ML tree (Fig. 1) and Bayesian tree (data not shown). Almost all nodes in the ML

Table 3. Estimated divergence times for each node in millions of years

Node	Age
an1	27-43
an2	38-60
an3	50-79
an4	238-380
an5	6-9
an6	19-30
an7	70*
an8	33*
an9	55*
an10	70-108
an11	94-148
an12	16-26
an13	71-114
an14	105-168
an15	192-305
an16	240-450

Ages of root and the next two deeper nodes from which the order Actiniaria and Zoanthiniaria descend are not shown because of inaccuracy of method in determining age of root and a low ML bootstrapping support on those nodes. *Calibration points used in determining divergence times, which corresponds to three nodes of phylogenetic tree in Fig. 1.

tree were highly supported by ML bootstrapping and Bayesian posterior probabilities (BPP).

The ML tree showed that the two representative orders (Alcyonacea and Gorgonacea) of the subclass Octocorallia are not monophyletic group. The temperate soft coral *D. gigantea* was clustered with *Pseudopterogorgia bipinnata* (Gorgonacea: Gorgoniidae), instead of being in sister relationship with *Sarcophyton glaucum* (Alcyonacea: Alcyoniidae) (Fig. 1). High ML bootstrapping values and Bayesian posterior probabilities strongly suggest that molecular data do not support the conventional taxonomic system in this subclass that is mostly based on morphological similarity. Previous studies showed a similar incongruence between taxonomic system and molecular phylogeny particularly at an ordinal level and a lack of resolution at the base of octocorallian phylogenetic tree. Recently McFadden et al. (2006) found that polyphyletic relationships are widely spread in the subclass Octocorallia, especially in five orders (Alcyonacea, Gorgonacea, Stolonifera, Telestacea, and Protoalcyonaria). Previously these orders were proposed to be combined in one order, Alcyonacea (Bayer, 1981) and this revision has been widely accepted (Fabricius, 2001). On the other hand a lack of resolution at deep nodes was interpreted as a possible result of radiating evolution among major lineages of octocorallians (McFadden et al., 2006). The radiation hypothesis waits further examination and supporting by different markers. Like the tree Medina et al. (2006) found, our tree reconfirmed that the corallimorpharians were nested within scleractinians. Based on this finding, it was argued that the taxonomy of scleractinian based on skeleton does not define a natural group and scleractinian skeletons might be lost in the nonskeletonized corallimorpharian lineage (Medina et al., 2006). However this hypothesis was not upheld in other study by Fukami et al. (2008). Based on phylogenetic analyses of extensive taxon sampling and different molecular data of nuclear genes (ribosomal DNA and β-tubulin) and mitochondrial genes (CYTB and COI), they found that the corallimorpharians and scleractinians are monophyletic group, respectively (Fukami et al., 2008). So this conflict warrants further investigations. For example, another phylogentic analysis using mtDNA genome data from the same sampling scheme used in Fukami et al.'s (2008) analysis might resolve the conflict, although not all the taxa are needed. Mitochondrial genome data from anthozoans does not yet reach that extent.

The ML tree topology in Fig. 1 is the same as Medina et al.'s (2006) except for the zoanthiniarian, *Palythoa* sp. According to Medina et al. (2006) this species was clustered to actiniarians with a low BPP value (84% and less then 50 % in ML bootstrapping). Our result showed a relatively close relationship of zoanthiniarian to octocorallians than to actinarians, although the node still has low ML bootstrapping value (79%). It is likely that a difference in aligned data set for analyses between studies might be responsible for the different results. In addition, only two DNA genes are usable for *Palythoa* sp. (Table 1). So uncertainty on the position of *Palythoa* sp. still remains.

We estimated divergence times of each node using the ML tree and the penalized likelihood method implemented in R8S program. As Medina et al. (2006) used two calibration schemes which were based on fossil records, we employed these two calibrations separately in the estimation for all the nodes except for the root and the next two nodes connected to the branch having low (79%) ML bootstrapping value. Ranges of divergence time for each node represent each result from the two calibration schemes (Fig. 1, Table 3). The basal *Astrangia* node (70 Ma) in the "Robust/Short" clade consistently resulted in more elevated values than when the other nodes in "Complex/Long" clade were used. In overall as nodes go back to deep along the tree, the range becomes wider. Therefore a careful interpretation is needed for such values from deep nodes. Our estimates are slightly different from those of Medina et al. (2006). The difference mainly occurred around upper boundary of ranges (Table 3). Our rooting method is based on midpoint rooting which defines a root in the middle of the longest branch connecting any two terminal taxa. This was inevitable in our analysis because of absence of relevant outgoup to the ingroup. On the contrary, Medina et al. (2006; see Fig. 1) used the octocorallians as outgroups to estimate diverging ages of two deeper nodes among the hexacorallians. Therefore it appears that the midpoint rooting is related to our elevated estimates of divergence times. Molecular dating of divergence time has been known to have various sources of errors including phylogenetic uncertainty, substitutional noise, and lineage effects (Sanderson and Doyle, 2001).

According to our analysis the octocorallians seem to start diverging around end of or after Cretaceous period (50-79 Ma). This age relatively very early compared to the long history of stony sea corals (>240 Ma). However because the estimate is based on only four taxa of the Octocorallia, whether the octocorallians really diversified and radiated widely in such a recent past remains to be tested. Thus, it will be interesting how the result will be when octocoralian taxa are comprehensively examined. Since mitochondrial genome data from the subclass Octocorallia is very scarce up to date, the sequence information of *D. gigantea* can be used as an useful molecular and comparative framework for another mitochondrial genome sequencing and constructing a more comprehensive phylogeny of the soft coral, octocorallians.

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